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Vaccine against Oncovirus Infections, such as infections by Feline Leukosis virus of the cat

Description

The invention concerns a vaccine on the basis of DNA, by which cats can be protected against infections with Feline Leukosis virus.

Feline Leukosis virus (FeLV) is a cat-specific, world wide endemic virus that causes severe disease and is one of the main causes of mortality in feline populations. Currently, the infection rate is between 12% to 16% in cats both in Europe and the USA.

Some of the cats can surmount the infection; however a life long persistence of virus in the organism is also possible. Latently infected cats are then deemed to be a reservoir of pathogens.

An efficacious therapy of FeLV infections leading to eradication of the disease is currently not possible. The best success currently is to push back the disease for some time. Certain chemotherapeutic agents can be applied to cats; side effects however are highly problematic, as they are in human medicine. The treatment of interferons is experimental currently.

Virostatic drugs are not capable to inactivate and thus also do not lead to a success definable as healing.

An effective management of FeLV infections can only be effected preventively by vaccination.

State of the art

Vaccines that are currently available either are based on inactivated FeLV viruses, on proteins produced by recombinant methods, so-called subunit vaccines, or on the use of genetically modified live vaccines. These classes of vaccines, however, show a number of disadvantages, apart from an unsatisfactory success of vaccination.

Preparations from inactivated viruses lead to the desired immunity only in a part of the vaccinated animals. These vaccines invariably consist of protein mixtures, in which highly immunogenic antigens have to compete with a lot of other proteins for the presentation by the immune system. Furthermore, after the vaccination strong side effects such as allergic reactions and autoimmune disease can occur.

A recombinant vaccine consisting of coat protein of FeLV produced by biotechnological methods, adjuvantated by aluminium hydroxide and saponin, is a vaccine frequently employed currently. Vaccination with this vaccine lead to protection against leukosis in 80% to 95% of the cats (Lutz et al., 1991, J Am Vet Med Assoc; 199(10): 1446-52).

A problem is the risk of the occurrence of fibrosarcoma at the site of vaccination. Another disadvantage of this vaccine is that the raised immunity is mainly based on the production of virus neutralising antibodies. Newer experimental results (Flynn et al., 2000, Immunology 101, 120-125) however show that for the formation of protective immunity, the cellular immune response is also of great importance.

The use of live vaccines has proven to be effective with regard to the effected immunity, however it contains the inherent risk that the virus strains employed convert to new pathogenic viral strains by mutation or recombination. Also there has to be observed that when using such vaccines that contain all viral structures, it cannot be discriminated after immunisation whether the animals were infected or vaccinated. For those two reasons, these vaccines are not suitable for practice.

Another example for a vaccine consisting of virus capable of infecting or replicating is a recombinant canary poxvirus expressing FeLV surface proteins. In experimental infections, 83% of the animals were protected from infection (Jarrett et al., 1993, J of Virology: 2370-2375). This vaccine however comprises the disadvantages of a live vaccine with regard to unpredictable recombinations; furthermore it is relatively difficult and hence, expensive to produce and to characterize.

Apart from such classic and modern recombinant vaccines, the possibility exists to vaccinate with DNA expression constructs. Only the information for certain immunogenic parts of the pathogen in the form of DNA is thereby given to the vaccinee. After vaccination, FeLV antigens are expressed by the cells of the vaccinated cat and stimulate an immune response against the virus in this way.

This possibility to attain an immune response against an antigen by injection of DNA expression constructs encoding this antigen was first published by Tang and Ulmer for the mouse (Tang et al., 1992, Nature 365, 152-154; Ulmer et al., 1993 Science 259, 1745-1749) and has been demonstrated in a great number of species since. It can be assumed that the general principle of vaccination with nucleic acids that encode immunogen is applicable to all

higher animals. Regarding the selection of suitable antigens, their encoding into nucleic acid sequences and the selection of a suitable vaccination regime, however, any application poses a number of problems some of which are difficult to surmount to the person skilled in the art, which has resulted in no DNA vaccine being admitted to testing in clinical phases 2 or 3, so far.

The vaccination of cats with expression constructs to express the genes env and gag is described in the French patent FR 2 751 223. The invention outlined therein however is purely hypothetical and not disclosed sufficiently; no expression- or immunisation experiments or results thereof are shown. It is a purely speculative application.

Experiments regarding DNA immunisation in the area of FeLV are known (Jarrett et al., 2000 Immunology 101, 120-125), however they did not lead to a convincing result. In this publication, the whole genome comprising a polymerase deletion was inoculated as an expression construct. The clinical success of the vaccination did not go as far as a protection of the cats against infection or viremia. Apart from such practical disadvantage of the cited vaccination experiment, the use of deletion mutants or their genome for vaccination has the disadvantage that the risk remains that novel infectious pathogens arise from recombination of a deleted virus with endogenous or exogenous viral sequences.

In contrary to the cited work by Jarrett, the aim of the efforts leading up to the present invention was to only express isolated FeLV antigens. Preliminary experiments however showed that inoculation of expression constructs that expressed homologous wild type sequences encoding the „env" and „gag" genes of FeLV under control of the cytomegalic virus (CMV) early immediate promoter did not provoke an antibody response in cats. Further experiments

also showed that the respective sequences were not expressed, or only to a very small degree, in human and feline cell lines. This phenomenon is known for sequences of the HI virus and other lentiviruses (Wagner et al., 2000, Hum Gene Ther 11(17), 2403-2413). The expression of wild type sequences in the infected cell is thereby dependent on the prior expression of the virally encoded rev protein.

Such expression control is not known for the FeLV virus, which does not belong to the class of lentiviruses, and a mechanism similar to "rev" control has never been demonstrated or postulated in the literature.

It is also known that by optimizing the expression construct's codon usage to the codons that are preferentially used in mammals, the expression of proteins can be significantly increased (Grantham et al., Nucleic Acids Res 1980, 9:1893-912). This method was already employed successfully to raise the expression level of various viral structural proteins of HIV-1 and SIV. The effect relies on the circumvention of "rev" dependent transport mechanisms for the extremely AT-rich transcript of these late proteins in the replication cycle of lentiviruses. Codon optimization of the DNA sequences of the "env" and "gag" protein of the human HI virus leads to far greater antibody titres against these synthetic antigens in mice than was possible for wild type sequences (Haas et al., 1998, J Virol. 72: 1497-503, Wagner et al., Hum Gene Ther. 2000,17:2403-13). The synthesis and use of such optimized sequences for vaccination against HIV-1 is also known from WO 00/029561 and WO 97/48370.

Another problem concerns the application of the DNA encoding the immunogenic antigens or parts thereof. A disadvantage of the vectors currently used for DNA transport (transfection) is caused by the fact that

either vectors of viral origin are used, which cause problems concerning the aspect of safety (Lehrman, 1999, Nature 401: 517-518), or plasmids are used. Since plasmids are produced by bacterial fermentation, they contain, apart from the desired gene, also DNA necessary for their propagation and selection and resistance genes against commonly used antibiotics. This problem is discussed in detail in WO 98/21322. It shall be mentioned that when using gene expression constructs on the basis of plasmid DNA, the inherent risk of propagating antibiotic resistance genes is present, which is especially intolerable in large vaccination campaigns.

Covalently closed minimalist DNA constructs such as disclosed in EP 0 914 318 B1 are a different type of DNA vector. Especially their application in the form of peptide-linked DNA constructs leads to a surprising, qualitatively improved immune response in comparison to unmodified DNA (also see DE 101 56 679.4 and DE 101 56 678.6).

Apart from the disadvantages caused by current gene transfer methods, an efficacious and safe vaccine against FeLV has not yet been developed. Until today, the treatment of a FeLV infection is restricted to a boosting of the unspecific defences of the animals and a treatment of secondary accompanying infections. The available vaccines comprise the side effects mentioned earlier.

Departing from this state of the art, it is the objective of the present invention to provide a vaccine, which leads to a protection of cats against infections with FeLV, as well as suitable diagnostic tools.

Solution of the problem and advantages of the invention

According to the invention, the objective is attained by immunizing cats with a mixture (cocktail) from synthetically made DNA sequences that are optimized for codon usage and splicing signals and encode structural proteins "gag" and the most important membrane protein "env" of FeLV.

In the course of optimisation regarding codon and signal usage, mutated sequences were obtained that led to the substitution of single amino acids in the structural proteins ("gag") and the most important membrane protein ("env") of FeLV. Surprisingly, these proteins with changed amino acid sequence provided the inventive advantages. In this regard, the optimisation of codon and signal usage according to the invention is a strategy for changing the amino acid sequence of the structural proteins "gag" and the most important membrane protein "env" of FeLV.

In the context of the invention, the following terms shall mean

„env":	Gene sequence encoding the viral coat proteins of the inner viral packaging of feline leukosis virus
„gag":	Gene sequence encoding the viral structural proteins of the inner viral packaging of feline leukosis virus
FeLV:	feline leukosis virus
WT:	wild type
WT „env":	wild type of the „env" DNA sequence, extracted

	from the NCBI database, Acc. No. M12500
WT „gag“:	wild type of the „gag“ DNA sequence, derived from the blood of infected cats (see example 1), no database sequence, but homologous to one.
NLS:	Nuclear localisation signal sequence
ODN:	oligodeoxynucleotide
PCR:	Polymerase Chain Reaction
LeadFeLVenv (FeLVenv):	FeLV “env” sequence with signal sequence
LeadFeLVenvgp85 (FeLVenvgp85):	FeLV “env” sequence (gp85) with signal sequence

The gene sequence “gag” encodes the viral structural proteins of the inner viral packaging, the gene sequence “env” encodes the coat proteins. The protein possessing the highest immunogenicity of all proteins encoded in the “env” sequence is the glycoprotein gp70. Virus neutralising antibodies are being produced in the cat organism against gp70. These antibodies constitute the first immune response after the entry of the pathogen into the body, which can in certain circumstances be sufficient to fight the infection.

Controversy exists whether membrane proteins or secreted proteins are better suited to induce virus neutralising antibodies. For this reason, two different constructs encoding “env” were made. It is known that the p15 sequence of the “env” gene sequence contains at least one sequence tract that has immune modulating properties (Haraguchi et al., 1997, Journal of Leukocyte Biology, 61, 654-666), by which it suppresses antibody formation. Hence, apart from a construct encoding gp70 and p15 (gp85), another

construct was made containing only gp70 and leading to the expression of a secreted "env" protein without transmembrane region.

A vaccine that can induce both virus neutralising antibodies against gp70 and a T-cell mediated immune response therefore constitutes a significant improvement in comparison to vaccines available to date, and might be employed also for the therapy of infected cats.

In order to express more antigen in-vivo and to elicit a stronger immune response that results in an effective and long-lasting protection against FeLV infection, the wild type sequences of "gag" and "env" were optimized. Optimization shall embrace both codon adaption and codon usage optimisation.

Every amino acid can be encoded by several codons. The frequency by which any particular codon is read during translation varies very substantially between viruses, bacteria and vertebrates. Accordingly, the occurrence of the respective tRNA in the cell also varies. Viral genomes show a codon usage frequency that partially differs from the host cell, which most probably comprises an element of expression control of the virus. By adapting the sequence to the host-specific codon usage pattern, such viral control mechanisms can be subverted and the expression of antigen can be increased substantially.

For this reason, the objective of the experiments was to attain a much stronger expression of the antigens by editing the viral sequences into sequences that represent a codon usage optimal for vertebrate genomes. A cloning strategy was developed with this objective which enabled the synthesis of such optimised DNA sequence from oligonucleotides.

Synthetic sequences were inserted into plasmids, propagated in E.coli, sequenced for control and subsequently transfected into a cat cell line in order to test the expression of the encoded proteins.

The proof of expression of the antigens was performed by Western blots.

The proof of expression of the proteins from the FeLV wild type sequences (WT) "env" and "gag" was performed by Western blot.

Both "env" and "gag" could be shown only as very small bands when assayed by immune precipitation. Surprisingly, this was also the case for the codon optimized "env" sequences. Only with "gag", codon optimization lead to a significantly improved expression, as shown in Fig. 1. After trying many other hypotheses that were meant to explain the dissatisfying expression of the synthetic genes, the synthetic "env" sequence was analysed for sites predicted by bioinformatics methods. A number of such signals predicted by the employed programme (Complign PPC, Mac Molly Tetra Version 3.10a (Softgene GmbH)) was deleted by point mutation, in order to verify the hypothesis that the presence of such structures inhibits the expression of the genes in the context of the promoter used. Surprisingly, it was possibly to demonstrate "env" as a strong protein band in the Western blot by this measure (see Fig. 2). The synthetic antigen sequences according to the invention showed the successful improved expression of the antigens by virtue of the strong bands.

Generally, a connection between the strength of expression of an expression system and the resulting strength of the immune response is postulated, although numerous findings suggest that neither a linear correlation exists, nor that necessarily every treatment with expression vectors confers

immunity in the desired strength (Wherry et al., Journal of Immunology 2002, 168 pp 4455-4461). For this reason, once the in-vitro expression results had been obtained, mice were immunised with peptide-linked expression vectors encoding the codon optimized and -signal optimized "env". The advantages of such peptide linked constructs in eliciting an immune response are explained in detail in the disclosures of EP 0 941 318 B1 and DE 101 56 678 A1. In order to determine the immunologic importance of the p15 protein of "env" with regard to the provocation of an immune response, both sequences encoding "env" according to the invention were used (Seq.ID 7, 8, 9 and 10). The sera of immunized mice were assayed for specific antibodies against the FeLV viral protein "env" by means of Western blot. The antibody levels after the second immunization in week 4 clearly demonstrate that the synthetic constructs lead to a strong stimulation of antibody formation in-vivo, as well. In comparison, five of six mice of group 4 showed a strong immune response against the inventive antigen sequence, whereas the WT sequence (group 1) only led to a weak immune response in two of six animals (see Fig. 3).

Plasmids can be used as DNA expression constructs, however according to the invention, minimalist immunologically defined expression constructs are preferred. These are linear, covalently closed expression cassettes that consist only of a CMV promoter, an intron, the respective gene sequence and a polyadenylation sequence. These covalently closed minimalist DNA constructs are referred to as Midge vectors in the following (MIDGE: MINIMALISTIC IMMUNOLOGICALLY DEFINED GENE EXPRESSION VECTOR); see EP 0 941 318 B1. The Midge constructs have the advantage that they allow the avoidance of structures that are not essential for their medical function, thus avoiding the disadvantages of the conventional gene transfer systems.

For transfection, biological, chemical and /or physical methods known in the state of the art can be employed, for instance transfection by ballistic transfer. In a preferred embodiment of the invention the transfection is achieved by intradermal injection by syringes or needle free injection devices.

The invention concerns expression constructs that lead to expression of antigens of FeLV in mammalian cells. The invention thus provides a vaccine that leads to protection from infection with FeLV in cats and considers the aspect of safety. According to the invention, conventional adjuvants are not employed, thus excluding the risk of fibrosarcoma formation at the site of injection.

Further advantageous methods are the biological transfection methods, such as peptide mediated gene transfer. To give an example, a DNA expression construct encoding at least the "env" and the "gag" sequence as provided by this invention, are attached covalently to a peptide, which preferably is the nuclear localisation signal (NLS) of the simian virus 40.

After the positive results in the mouse experiment, cats were immunized with the expression constructs and their antibody status was determined.

The attached sequence protocol, which is part of the application and the present disclosure, contains the following sequences:

Seq. ID	Sequence name/-description
Seq.ID1	DNA sequence of the "env"-gene wild type
Seq.ID2	DNA sequence of the "gag" gene wild type
Seq.ID3	Protein sequence of the "env"-gene wild type

Seq.ID4 Protein sequence of the "gag" gene wild type

Seq.ID5 DNA sequence of the mutated "gag" gene

Seq.ID6 Protein sequence of the mutated "gag" gene

Seq.ID7 DNA sequence of the mutated "env" gene (gp85). The gp70 sequence is extended here by the sequence encoding the immunogenic p15 protein.

Seq.ID8 DNA sequence of the mutated "env" gene (gp70)

Seq.ID9 Protein sequence of the mutated "env" gene (gp85)

Seq.ID10 Protein sequence of the mutated "env" gene (gp70)

Seq.ID11 DNA sequence of the wild type of the "env" gene (gp70), derived from Seq.ID1 (NCBI database, Acc. No. M12500).

Seq.ID12 to Seq.ID40 sequences of the primers employed according to the following examples.

According to the invention, a DNA expression construct is provided for the expression of gene products of the feline leukosis virus (FeLV) in cat cells, consisting of a promoter sequence operable in Felidae and at least one nucleotide sequence that is related to a wild type nucleotide sequence encoding an original structural protein ("gag") and /or a membrane protein ("env") of FeLV, wherein said nucleotide sequence of FeLV is mutated and contains no open or hidden donor and /or acceptor sequences or a highly homologous but not identical part thereof. The proteins, which are highly homologous but not identical to the original membrane protein ("env") of FeLV, show a homology to the corresponding wild type of at least 98%. Preferred is an expression construct containing the sequences Seq.ID5, Seq.ID7 and /or Seq.ID8.

The structural or membrane proteins are encoded completely or partially by the corresponding nucleotide sequences. The expression construct is either a plasmid or a construct in which the immunising polynucleotide sequences are in the form of expression constructs comprising a linear double stranded region and the single strands forming said double stranded region are attached to each other by short single stranded loops made of deoxyribonucleotides, and where the single strands forming said double strand only consist of the coding sequence, under control of a promoter sequence operable in the in the vaccinated animal, and a terminator sequence.

For an improved transfection, the expression construct can be attached to one or more peptides covalently. A peptide of 3 to 30 amino acids, at least half of which are taken from the group of arginine and lysine, especially a peptide with the amino acid sequence PKKKRKV (Proline-Lysine-Lysine-Lysine-Arginine-Lysine-Valine), is preferred.

According to the invention, also proteins are provided that are a protein highly homologous to the original structural protein („gag") of the Feline Leukosis virus (FeLV) (Seq.ID6) or with the original membrane protein gp85 („env") of FeLV (Seq.ID9) or with the original membrane protein gp70 („env") of FeLV (Seq.ID10). These proteins in turn can be used for antibody production (monoclonal or polyclonal antibodies), which again in turn can be part of diagnostic kits for the diagnosis of infection of cats with feline leukosis virus.

The expression construct according to the invention is provided as part of a pharmaceutical composition, especially a vaccine for the production of preventive and /or therapeutic immunity in felidae, especially in cats.

Further advantageous embodiments of the invention can be derived from the dependent claims and the description. The surprising effect of the pharmaceutical according to the invention as a vaccine for FeLV therapy and the inventive method is explained by the figures and examples. In this context, the abbreviations shall have the following meanings:

Midge-NLS-FeLVenvgp85(-) Midge-NLS-FeLVgp85(-)	or NLS-coupled Midge vector encoding the codon- and signal optimized "env" sequence with p15 (gp85)
Midge-NLS-FeLVenvgp70(-) Midge-NLS-FeLVgp70(-)	or NLS-coupled Midge vector encoding the codon- and signal optimized "env" sequence without p15 (gp70)
Midge-NLS-WT	NLS-coupled Midge vector encoding the WT of the "env" gene
mAK vs. gp70	Monoclonal antibody against gp70
Positive control	Leukogen vaccine
Buffer	PBS, negative control

It is shown in:

Fig.1: in-vitro comparison of expression of the WT "gag"-protein and the codon optimized "gag" protein. Loaded was lysate from cat cells that previously had been transfected with the following constructs. The "gag" precursor has a size of 55 kD:

Lane 1 + 2: expression constructs encoding "gag"-WT
Lane 3: expression vectors encoding codon-optimized "gag"
Lane 4: non-infected cat cells, negative control
Lane 5: empty
Lane 6: virus-infected cat cells, positive control

Lane 7: Boa protein marker

Expression by WT leads to very weak protein bands (1 and 2), whereas a strong expression is attained by the sequence according to the invention (3). Infected cat cells served as a comparison (6). „gag" signifies a precursor protein that is cleaved into structural proteins of the FeLV in the infected cell by means of proteases. The strongest immunogen is the structural protein p27, which is thus well recognized by an antiserum against whole virus. This explains why in lane 6, both a band representing the 55kDa whole "gag" and a 27-kDa band can be recognized. In contrast to this situation, lane 3 does not contain virus particles, but cells transfected with "gag" gene; this apparently does not lead to a degradation of the "gag" gene product into the viral proteins by means of proteases, but to the unspecific degradation of the entire precursor protein by cellular proteases.

Fig.2: in-vitro comparison of expression of the WT "env" gene and the codon- and -optimized "env" sequence (gp85). Loaded was lysate from cat cells that previously had been transfected with the following constructs:

Lane 1 + 2: expression constructs encoding "gag"-WT

Lane 3: expression constructs encoding codon optimized "gag"

Lane 4:

Lane 5: empty

Lane 6:

Lane 7: Boa protein marker

Lane 1: Boa protein marker

Lane 2: non-infected cat cells, negative control

Lane 3: virus-infected cat cells, positive control

Lane 4: empty

Lane 5: virus-infected cat cells, precipitate, positive control
Lane 6: non-infected cat cells, precipitate, negative control
Lane 7: further negative controls for specific proof of "env"
Lane 8: empty
Lane 9: expression vectors encoding codon- and signal optimized FeLVenvgp85(-)
Lane 10: empty
Lane 11: expression vectors encoding codon-optimized FeLVenvgp70(-)

The control in lane 5 provides a clear protein band that can be used as a positive control for the expression of envgp85. Lane9 shows that the inventive sequence FeLVenvgp85(-) induces the expression of the gp85 protein. Lane 11 shows the inventive FeLVenvgp70(-). The absence of the 70kDa band can be explained by the envgp70 being a secreted protein, in contrast to gp85, and thus being difficult to detect, if at all, in the cell lysate.

Fig.3: in-vivo results after immunisation of mice with expression constructs encoding "env". The abbreviations have the following meaning:

A: Positive control
B: buffer

Antibody measurement was performed at week 4 after the second immunisation. In group 3, 3 of 6 mouse sera are antibody positive (gel lanes under the arrow: Midge-NLS-FeLVgp85(-)), in the 4. group, 5 sear test strongly positive and 1 weakly positive (gel lanes under the arrow: Midge-NLS-FeLVgp70(-)). The animals immunized with WT in group 5 however showed very weak positive signals in only two cases (gel lanes under the arrow: Midge-NLS-WT). The experiment

demonstrates that the optimized sequences lead to a much improved antibody formation in vivo also, in comparison to the WT sequences, and confirms the results of the in-vitro experiments.

Fig. 4: DNA sequence comparison of the wild type "gag" gene (Seq.ID2) against the codon-optimized "gag" gene (Seq.ID5). Similarity: 74.51%

Fig. 5: DNA sequence comparison of the wild type „env" (gp70 region from Seq.ID1) against the codon- and signal optimized „env" gene (gp70; Seq.ID8). Similarity: 75.75%

Fig. 6: DNA sequence comparison of the wild type „env" gene (Seq.ID1) against the codon- and signal optimized „env" gene (gp85) (Seq.ID7). Similarity: 80.25%

Fig. 7: Protein sequence comparison of the wild type "gag" protein (Seq.ID4) against the protein sequence of the codon-optimized "gag" protein (Seq.ID6). Similarity: 98.62%

Fig. 8: Protein sequence comparison of the wild type „env" protein (Seq.ID3) against the protein sequence of the codon- and signal optimized „env" protein (gp70) (Seq.ID10). Similarity: 98,75%

Fig. 9: Protein sequence comparison of the wild type „env" protein (Seq.ID3) against the protein sequence of the codon- and signal optimized „env" protein (gp85) (Seq.ID9). Similarity: 98.60%

The results show that the codon- and signal optimized DNA sequences both of the "env" and the "gag" gene of FeLV show a homology to the corresponding wild type of at least 74%. From these arise protein sequences of the "env" and "gag" protein of at least 98%. Other optimizations of the DNA sequence of "env" and "gag" of FeLV are conceivable that lead to a similar

result, that is a high degree of homology between the original wild type and the protein sequence resulting from the optimisation. Such optimisations are also to be understood to be within the scope of the invention.

Example 1: Wild type (WT)-sequences

The wild type sequences of the selected antigens, especially the "gag" gene, were obtained from the blood of infected cats. The DNA sequence of the "env" WT is given in Seq. ID 1 (NCBI data base, Acc. No. : M12500), for the "gag" WT in Seq. ID 2. The corresponding amino acid sequences are given in Seq. ID 3 ("env") and Seq.ID 4 ("gag").

Primer for "gag" WT:
In order to remove two Eco31I restriction sites, 3 PCR with the following mutation primers were performed:

gag-mut1-rneu (Seq.ID12):
AATTAAGAGCTCCACGTCTCCCCCCGCTAACAGCAACTGGCG

gag-mut2-l (Seq.ID13):
AATTAAGAGCTCCAGGTCTCCGGGGCTCCGCGGGGCTGCAAGACG

gag-mut3-r (Seq.ID14):
AATTAAGAGCTCCACGTCTCCTTCCCTTTTGTTGTATATCTTTTCTGC

gag-mut4-l (Seq.ID15):
AATTAAGAGCTCCAGGTCTCCGGAAACCCCAGAGGAAAGGGAAGAAAG

After ligation of the three sequences thus obtained, a PCR was performed with the primers:

Felvgag-l (Seq.ID16):

CGGATAAGGTACCATGGGCCAAACTATAACTACC

Felvgag-r (Seq.ID17):

TTCTCAGAGCTCTTAGAGGAGAGTGGAGTTGGCGGGT

Primer for "env" WT:

envl (Seq.ID.18): CGGATAAGGTACCATGGCCAATCCTAGTCCACC

envr (Seq.ID19): AGTTCTCAGAGCTCTTAGGCTGTTTCAAGGAGGGCTT

Example 2: codon optimization

The codon usage table for cats was obtained from the codon usage database (http://www.kazusa.or.jp/codon/). For every amino acid of the two WT sequences, the codon used most frequently was employed. The following restrictions to this rule were used:

If an amino acid was present more than three times in sequence, from the fourth one onwards, the second most frequent codon was used. This was meant to avoid the sudden extreme depletion of the tRNAs and to assure the efficacy of transcription.

In order to avoid uncontrolled immune stimulatory effects, sequences with a C and immediately following G were avoided. In order to avoid restriction sites for Eco31I, KpnI and SacI, all sequences of the base sequence GAGCTC, GGTACC, GGTCTC and GAGACC were removed by choosing alternative, also frequently used codons.

Example 3: Cloning of FeLVenv

Oligonucleotides of a length between 18 and 28 bases were purchased (TibMolbiol). In total, 51 oligonucleotides were joined by annealing and ligation. The overlap was 4 bases. It was assured that any overlap occurred only once and was not palindromic. Every single oligonucleotide was hybridized strand against antisense strand, by heating the single strands (strand and antisense strand) in kinase buffer to about 80 degC and then cooling them slowly to room temperature. Afterwards, ATP and polynucleotide kinase (PNK) and oligonucleotides were phosphorylated for one hour. Then, in a first step, oligonucleotides neighbouring each other in the sequence were brought together and ligated (oligonucleotide 1+2, oligonucleotide 3+4). After 1 h of ligation, an aliquot of the ligation reaction of oligonucleotides 1+2 and an aliquot of the ligation reaction of 3+4 was brought together. An aliquot of this last ligation reaction was taken and a PCR with flanking primers was performed. If a PCR product of the expected length was obtained, it was inserted into the TOPO Vector pCR 2.1 by TA cloning and the sequence was verified. This was performed analogously for all other fragments of the complete gene. 4 fragments were obtained. The individual fragments were excised from the plasmid of control insertion by EcoRI and ligated after digestion with Eco31I. The complete ligation product of correct length was digested with BamHI and SacI and inserted into the similarly prepared vector pMCV1.4 after gel extraction. Subsequently, the sequence was verified by sequencing. The resulting plasmid was designated pMCV1.4-FeLVenv.

Primer sequences for the 4 assembled fragments:

Fragment 1:

left primer (Seq.ID20): ATATTGGATCCCATGGCCAACCCCTCCC

right primer (Seq.ID21) ATTATGGTCTCCTGCTGCTTCTTCCTGTCTGTGG

Fragment 2:

left primer (Seq.ID22): TAATAGGTCTCCAGCAGCAGACCTACCCCT

right primer (Seq.ID23): TAATAGGTCTCTGTGAACAGGGCAATGGGGTCA

Fragment 3:

left primer (Seq.ID24): TATTTGGTCTCTTCACAGTGTCCAGGCAGGTGTC

right primer (Seq.ID25): TATTAGGTCTCAGCTTGTGCTGGGGGGTGG

Fragment 4:

left primer (Seq.ID26): AATAAGGTCTCCAAGCTGACCATCTCTGAGGTGT

right primer (Seq.ID27): ATTAAGAGCTCTCAGGCTGTTTCCAGC

total sequence:

left primer (Seq.ID20): ATATTGGATCCCATGGCCAACCCCTCCC

right primer (Seq.ID27): ATTAAGAGCTCTCAGGCTGTTTCCAGC

Example 4: cloning strategy for LeadFeLVenv

For successful processing of the "env2 protein, a signal sequence (leader sequence) was inserted in front of the codon optimized "env" sequence. This signal sequence was assembled from a total of 8 ODN with a length of between 22 to 30 bp by annealing and ligation. From the last ligation step, a PCR was performed for amplification of the leader sequence.

Primer sequences for the complete signal sequence:

left primer (Seq.ID28): ATTGCCGGTACCATGGAGTCCCCCACCCACC

right primer (Seq.ID29): ATCAGAGGTCTCCCATGCCAATGTCAATGGTGAAC

At the 3`-end of the PCR product, an Eco31I recognition sequence was generated, which led to an overhanging end after digestion that was reverse complementary to the 5' end of an overhang generated by a similar digestion of the following PCR product.

PCR for FeLVenv:

On the 5' end of the sequence, an Eco31I recognition sequence was generated.

Employed primer sequences:

left primer (Seq.ID30): GATCTGGGTCTCCATGGCCAACCCCTC

right primer (Seq.ID27): ATTAAGAGCTCTCAGGCTGTTTCCAGC

After digestion of the two PCR products with Eco31I, these were purified and ligated to each other. The ligation product was further processed in a PCR, in

which a recognition sequence was generated for KpnI at the 5'-end and for SacI at the 3'-end.

Employed primer sequences:

left primer (Seq.ID28): ATTGCCGGTACCATGGAGTCCCCCACCCACC

right primer (Seq.ID27): ATTAAGAGCTCTCAGGCTGTTTCCAGC

The PCR product was digested with KpnI and SacI and inserted into the similarly digested vector pMCV1.4. The resulting plasmid was denominated pMCV1.4-LeadFeLVenv.

Example 5: cloning strategy for LeadFeLVenvgp85

The complete "env" polyprotein consisting of gp70 and p15 was cloned. To this end, the p15 WT sequence was amplified by PCR from the plasmid pMCV1.4-FeLVenvp15 and und inserted behind the pMCV1.4-LeadFeLVenv as above mentioned above.

In the amplification of the p15 an Eco31I recognition sequence was generated at the 5`-end.

1. PCR:

Employed primer sequences:

left primer (Seq.ID31): AATTATGGTCTCGCAGTTCAGACAACTACAAATGGC

right primer (Seq.ID32): AATTATGAGCTCTCAGGGCCTGTCAGGGTC

2. PCR:

The sequence of LeadFeLVenv was amplified. Thereby, a recognition sequence was generated at the 3`-end.

Employed primer sequences:

left primer (Seq.ID33): AATTATGGTACCATGGAGTCCCCCACCC

right primer (Seq.ID34): TATAATGGTCTCAACTGGGCTGTTTCCAGCAGGGC

After digestion of the two PCR products with Eco31I these were ligated to each other. The ligation product was processed in a PCR with the following primer sequences:

left primer (Seq.ID33): AATTATGGTACCATGGAGTCCCCCACCC

right primer (Seq.ID32): AATTATGAGCTCTCAGGGCCTGTCAGGGTC

At the 5´ end, a KpnI recognition sequence was thus generated, and a SacI recognition site at the 3`end. After digestion of the PCR product with KpnI and SacI, it was ligated into the similarly digested pMCV1.4 and cloned. The resulting plasmid was designated pMCV1.4-LeadFeLVenvgp85.

Example 6: Splice-Signal optimization of LeadFeLVenvgp85 (-splice):

The DNA sequence of LeadFeLVenv was analysed for possible splice signal sequences (sites) according to http://www.fruitfly.org/seq_tools/.html.
Between bases 100 and 140, a site was recognized with 97% probability.
After exchanging of base 119 (A to G, amino acid exchange from Gln to Arg),

no more potential sites were recognized (lower threshold = 40% probability). The generation and cloning of the mutated sequence was performed as follows:

PCR for the generation of the mutated sequence:

First, a fragment (1) was amplified by means of PCR, consisting of bases 1 – 123 of LeadsynFeLVenv. The forward primer employed generates the recognition sequence for the restriction enzyme KpnI at the 5' end of the PCR product.

The sequence of the reverse primer was chosen so that the PCR product contained the mutation (base 119=G). Additionally, the PCR generated the recognition site of the restriction enzyme Eco31I at the 3'-end of the PCR product. Generally, Eco31I generates a 4 base 5'overhang of the bases 2-5 downstream of the recognition sequence.

The 4-base 5'-overhang generated by digestion of the PCR product by Eco31I at the end of fragment 1 corresponds to bases 120 –123 of the sequence of LeadFeLVenv. This sequence in turn corresponds to the overhang generated by digesting LeadFeLVenv with the restriction enzyme BgLII, since bases 119 – 124 of LeadFeLVenv constitute the recognition site of BgLII.

Bases 1 – 123 are excised from the construct pMCV1.4-LeadFeLVenv by KpnI and BgLII.

After digestion of the PCR product fragment 1 (with mutation of the base 119=G) with KpnI and Eco31I, this can be ligated and cloned into the vector pMCV1.4-LeadFeLVenv, which had previously been digested with KpnI and

BgLII, and purified. The resulting plasmid was designated pMCV1.4-FeLVenvgp70 (-splice).

Employed primer sequences:

left primer (Seq.ID28): 5`-ATTGCCGGTACCATGGAGTCCCCCACCCACC

right primer (Seq.ID35): 5`-ATATTAGGTCTCAGATCCGGGGGGGGGAGGG

Analogously, the PCR product fragment 1 was ligated cloned into the vector pMCV1.4-LeadFeLVenvgp85(-splice) which had previously been digested with KpnI and BgLII, and purified. The resulting plasmid was designated pMCV1.4-FeLVenvgp85(-splice).

Example 7: cloning strategy for FeLVgag:

Cloning of FeLVgag was performed analogously to the process described under 3. The sequence was produced by annealing and ligating oligonucleotides to three single fragments. The sequence was assembled from a total of 2 x 31 oligonucleotides (forward and reverse strand). The fragments were used as templates in a PCR and amplified with the following primer sequences:

Fragment 1:

left primer (Seq.ID36): ATATTGGTCTCAGGAGAGGGACAAGAAGAG

right primer (Seq.ID37): AATATGGTCTCTCAGCCTGCTGGCGATGGGGC

Fragment 2:

left primer (Seq.ID38): ATTATGGTCTCTGCACCTGAGGCTGTACAGGC

right primer (Seq.ID39): AATATGGTCTCGGTGCTCCCTGCCGGCGGGGGTGCA

Fragment 3:

left primer (Seq.ID38): ATTATGGTCTCTGCACCTGAGGCTGTACAGGC

right primer (Seq.ID40): AATATGGTCTCTCTCCTCCTGCCTCTGC

Primer for the complete fragment:

left primer (Seq.ID36): ATATTGGTCTCAGGAGAGGGACAAGAAGAG

right primer (seq.ID40): AATATGGTCTCTCTCCTCCTGCCTCTGC

Fragment 1, 2 and 3 were cloned into the TOPO-Vector pCR 2.1.intermediately, digested subsequently with Eco 31I and extracted. The ligation product from 1,2 and 3 was digested with KpnI and SacI and cloned into pCMV1.4. The resulting plasmid was designatedpCMV1.4-FeLVgag.

Example 7: Transfection of cells, proof of expression:

Feline cells of the f3201 cell line were transfected with the plasmids pMCV1.4-FeLVenvgp85(-splice), pMCV1.4-FeLVenvgp70(-splice), FeLVgag and the WT containing plasmids pMOK for "env" and "gag" by means of electroporation at 250 V and 1050µF.

The expression of proteins was demonstrated by the Western blot method. Monoclonal mouse antibodies were employed in the assay.. Positive control: FeLV A infected cells of the f3201 cell line.

Example 8: production of peptide-linked Midge:

The plasmids pMCV1.4-FeLVenvgp85(-splice), pMCV1.4-FeLVenvgp70(-splice) and pMCV1.4-FeLVgag were digested to completion with the restriction enzyme Eco31I overnight at 37°C. Two DNA fragments were generated by the restriction digest. One consisted of the kanamycin resistance gene, and other sequences necessary for the propagation of the plasmid in bacteria. The other fragment consisted of the sequences that were to be part of the MIDGE-DNA: enhanced CMV-Promoter, chimeric Intron, the corresponding gene sequence and the polyadenylation sequence of SV40.

5'-phosphorylated hairpin oligonucleotides (TIBMolBiol, Berlin) 5' -PH-GGGAGTCCAGT xT TTCTGGAC -3' and 5' PH- AGG GGT CCA GTT TTC TGG AC-3 were ligated to the MIDGE-forming DNA fragment by means of the enzyme T4-DNA-Ligase in the presence of the restriction enzyme Eco31 I overnight at 37°C. The reaction was stopped by heating to 70 °C. The resulting mix of nucleic acids was treated with the enzyme T7-DNA-Polymerase. The Midge DNA was purified by anion exchange chromatography and precipitated by isopropanol (see EP 0 941 318 B1)

Production of the peptide-linked ODN:

The NLS peptide PKKKRKV was linked to the ODN in two steps. Firstly, the modified oligonucleotide 5'-PH-d(GGGAGTCCAGT xT TTCTGGAC, where xT is an amino-modified thymine base with a C_2 – amino linker) (0,1mM) was activated by sulfo-KMUS (5mM) in PBS at room temperature (RT). The

reaction was stopped by adding 50 mM Tris-(Hydroxymethyl)-Aminomethane after 120 min, and the activated ODN was obtained after ethanol precipitation (300mM NaOAc pH 5.2, 5.5 mM $MgCl_2$, 70 % Ethanol), centrifugation and a single wash with 70% ethanol. The ODN (0,1mM) thus obtained was dissolved in PBS and reacted with the peptide (0,2mM) for one hour at room temperature. The reaction was checked by gel electrophoresis (3%) and ethidium bromide staining. The resulting NLS-linked ODN was purified by HPLC and used for the synthesis of the MIDGE-NLS-constructs as described above.

Example 9: Antibody assay in mice

Five vaccination groups at six BALB/c mice each were formed (see table 1). The basic antigen in all groups (except the control groups) were the optimized sequences of the "env" protein with and without the immune modulating protein p15. The coding sequence and the cytomegalic virus promoter (CMV) preceding the sequences are employed as linear double stranded molecules according to example 8. As controls, buffer, the common vaccine (Leukogen) and the WT of the "env" protein were employed. After the first immunisation (50 µg DNA, 1mg/ml, i.d.), a second immunisation (boost) was performed on the 15th day. Blood was taken on days 14, 28 and 42. The blood samples were assayed for specific antibodies against "env".

Table 1: Composition of vaccinee groups

Gr.	Mice	Antigen used	Function
1	6	Leukogen	Positive control
2	6	PBS buffer	Negative control

3	6	FeLVenvgp85(-splice)	Determine antibodies
4	6	FeLVenvgp70(-splice)	Determine antibodies
5	6	WT "env"	Positive control

The results are shown in Figure 3.

Example 10a: Immunisation of cats

In order to determine whether the synthetic sequences are able to elicit a humoral and cellular immune response in cats, the following vaccination regime was formulated (table 2):

Table 2: Composition of vaccinee groups

Gr.	Cats	Antigen used	Function
1	5	FeLVenvgp85(-splice) FeLVgag	Determine antibody and cytokine status
2	5	FeLVenvgp70(-splice) FeLVgag	Determine antibody and cytokine status, comparison to group 1
3	2	PBS Puffer	Negative control
4	3	Leukogen	Positive control

Cats of the first two groups are immunized twice with a total of 600 µg DNA each, dissolved in PBS buffer. The peptide-linked expression constructs are applied by intradermal injection into the neck. The immune response is followed over 12 weeks, The secondary immunisation was performed in

week 4. From determining the cytokine status from weekly sampled blood samples, clues about the direction of the immune response (Th1, Th2) shall be derived. IL-4 was deemed to be an indicator of a TH2 response; IL-2 and Interferon-gamma were deemed to be indicators of a predominantly TH1 immune response. The vaccine Leukogen contains recombinant "env" protein and is used as a positive control.

Antibodies against the employed antigens were determined by means of Western blot and ELISA.

The amount of mRNA encoding the cytokines IL-2, IL-4 and Interferon-gamma was determined by means of real-time PCR.

Example 10b: *in vivo* results after immunisation of cats according to the vaccine regime described in table 2.

Semi-quantitative assay of antibodies against the "env" protein was performed by means of Western blot. Plasma samples of cats from experimental week 0 and 12 were tested. In week 0, no antibodies against "env" could be found, according to expectations. The weak bands mentioned in table 3 as (+) are unspecific. In week 12, all animals of groups 1, 2 and 4, with the exception of one cat, showed a clear antibody response. These results in the patient animal corroborate those of the pre-experiment in mice (see Example 9).

Herein signify:	+++	very strong band,
	++	strong band,
	+	visible band,
	(+)	weak band.

The strength of the bands represent the concentration of the antibodies in the plasma of the immunized cats.

Table 3: Determination of the humoral immune response against the FeLV protein

Gr.	Employed antigen	cats	Week 0	Week 12
1	FeLVenvgp85(-)	1	-	+++
	FeLVgag	2	-	++
		3	-	++
		4	-	++
		5	-	++
2	FeLVenvgp70(-)	1	-	+++
	FeLVgag	2	(+)	+++
		3	-	++
		4	-	++
		5	-	+
3	PBS buffer	1	(+)	-
		2	(+)	(+)
4	Leukogen	1	-	+++
		2	-	+++
		3	-	(+)

Example 11: Infection as control of the protection conferred by vaccination

In order to control whether the attained high antibody production is really conferring protection against infection by FeL-Virus, and thus to check the efficacy of the vaccine according to the invention, an experiment with infection followed. Four groups of 10 cats each were vaccinated twice (day 0 and day 21) with the respective constructs intradermally with a needle-free injection device (table 4). As expression constructs, NLS peptide-linked Midge vectors were used. On day 21, 22 and 23 after the last vaccination, cats were infected by a trial infection with live virus (Rickard strain, > 10e6 focus-forming units/ml). The efficacy of the vaccine was determined according to whether the cats were protected after the trial infection. Cats were deemed protected if they had no virus particles in their blood (seronegative cats) and no viral DNA in their blood cells. In order to assay the virus particles, cat serum was tested for presence of antigen p27 by ELISA. The amount of integrated viral DNA, the so-called proviral DNA, was assayed by Taqman PCR. The following vaccination regime was formulated:

Table 4:

Gr.	Antigen employed	DNA-Doses [µg]	Number of seronegative cats 105 days after trial infection
1	PBS buffer	-	0
2	FeLVgag	2 x 100	2
3	FeLVenvgp70(-splice)	2 x 50	4

PBS buffer was used as negative control.

The results can be summarised as follows:

Table 4 shows the results of the serum assay of the cats regarding the presence of virus protein p27. This test is a generally accepted assay for the diagnosis of FeLV viremia. In parallel, white blood cells of the same cats were analysed for the presence of proviral DNA (data not shown). All virus protein-free cats were also free of proviral DNA.

Since the two test systems determine different steps of the viral development in the body, the twofold negative result indicates that the virus was not able to multiply in the animals' bodies, which is equivalent to protection.

In groups 2 and 3, some of the cats could be protected against the infection with FeLV.

In group 2, 2 of 10 cats were free both of virus protein and of proviral DNA. This vaccine protection was based on the application of the inventive vaccine (Seq.ID 5).

In group 3, 40% of the animals could be protected by the inventive vaccine (Seq.ID 8) against infection with the FeL-Virus. This is a significant reduction of infected cats in comparison to group 1.

In the animals, neither viral protein nor proviral DNA could be detected in the serum and blood samples. It is notable that in order to attain protection in the 4 cats of group 3, the very small dose of 50µg per injection was sufficient in order to protect the cats. That is of advantage since the DNA concentration to be applied is small and hence, the production costs of the vaccine are decreased rapidly.

All animals of group 1 (control group) showed viral particles in their blood, i.e. they were not protected and fully receptive for the trial infection.

During the entire immunization experiment, neither side effects in the form of local irritations at the sites of injection, nor disturbances in the general state of well-being of the experimental animals were observed.